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Hydrophobic Radicals Embedded in Neutral Surfactants for Dynamic Nuclear Polarization of Aqueous Environments at 9.4 Tesla.

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We show how large DNP enhancements of NMR signals can be obtained from several hydrophobic radicals that are solubilised in aqueous environments by a variety of biologically compatible neutral amphiphiles. In particular we show that the bi-radical TEKPOL can be incorporated into micelles formed by the surfactant polysorbate 80 (Tween-80), where we obtain large DNP enhancements (~60) at 9.4 T and ~100 K.

Dynamic nuclear polarization (DNP) provides a way to significantly enhance the signal intensity in NMR experiments. It works by transferring the high polarization of unpaired electrons to nearby nuclei of interest. Typically, the source of electron spin polarization is introduced into otherwise diamagnetic samples by adding a paramagnetic polarizing agent that is often a stable organic radical such as a nitroxide or a trityl radical derivative.[1]

Transfer of spin polarization from the electrons to the nuclei is driven by irradiation of electron-spin transitions in the microwave region. The nature of the polarizing agent and its electronic properties (EPR frequencies, electron relaxation times, electron-electron dipolar couplings, etc.) are central to obtaining large DNP enhancements. Since acquisition times in NMR experiments decrease as the square of the signal intensity, DNP enhancements on the order of 100 of more potentially accelerate NMR experiments by four orders of magnitude, making a range of previously impractical applications accessible.[1e,4b,2] As a result, there have been extensive recent efforts to develop high-performance polarizing agents, both for very low temperature DNP (<4 K),[1d,3] and for *in situ* magic angle spinning DNP experiments at ~100 K.[1c,e,4] For MAS DNP, over the last few years, by engineering the radical properties,[5] it was possible to increase the ^{1}H DNP enhancement factor at 400 MHz (9.4 T) and ~105 K from around 20 for the mono-radical TEMPO,[6] to ~40-60 for the bi-radical TOTAPOL,[1c,7] and then to more than 200 for the bi-radical TEKPOL[4d] (which has long electron relaxation times). Other bi-radicals for so-called Cross-Effect (CE)[8,1e] DNP include bTbK,[4a] bCTbK,[4b] and AMUPol.[4e] However, many of the best radicals are hydrophobic, and are therefore not soluble in aqueous solvents, which means they cannot be used notably for biological applications. The requirement that the radical be water soluble poses additional constraints on design strategies, necessitating the incorporation of hydrophilic groups[4c,e,9] or the use of host-guest complexes.[10]

Most solid-state DNP MAS NMR experiments are performed at ~100 K, where the polarizing agent is dispersed in a frozen glassy matrix. For biological samples this matrix is generally composed of a deuterated glycerol/water mixture (d_8-Glycerol:D_2O:H_2O 60:30:10) that promotes glass formation. Currently, essentially only four biradicals: TOTAPOL,[1c] SPIROPOL[4c] and the recently introduced AMUPol and PyPol,[4e] are available for MAS DNP in biological environments, since most of the other radicals are insoluble in water.

In a very recent paper Kiesewetter *et al*,[11] showed that significant DNP enhancements can be obtained (at 5 T) by solubilizing the hydrophobic biradical bTbK[4a] in a water/glycerol mixture with the help of a deuterated surfactant, sodium octyl sulphate (d_{17}-SOS 95%). We have also been developing a similar approach, and here we show how several hydrophobic radicals can be solubilised in aqueous environments by a variety of biologically compatible neutral amphiphiles. In particular we show that the bi-radical TEKPOL can be incorporated into larger micelles formed by polysorbate 80 (Tween-80), where we obtain enhancements of ~60 at 9.4 T with protonated surfactant (as compared to ~40 for bTbK). This demonstrates that the approach is quite general. Such solubilisation of nitroxide mono-radicals into micelles has been investigated extensively in the past in order to characterize the dynamical behaviour of micelle phases by EPR.[12]

We observed that bTbK is easily solubilized in a concentration range of 10-15 mM by simply stirring powdered bTbK in water containing micelles formed with 100 mM sodium dodecyl sulphate (SDS). Figure 1 shows the ^{1}H and ^{13}C solution NMR spectra of an H_2O solution of glucose (50 mM) and with 100 mM SDS (the critical micelle concentration (cmc) for SDS is 8.3 mM). In Figure 1C,F the NMR spectra of the same solution but with the addition of 10 mM bTbK. The strong paramagnetic relaxation enhancement (PRE) induced by the nitroxide biradical selectively broadens beyond detection only the SDS resonances, but does not broaden the resonances of the glucose dissolved in the aqueous

phase, clearly showing that the radical is completely incorporated into the SDS micelles, and that there is a negligible amount of free-radical in solution.

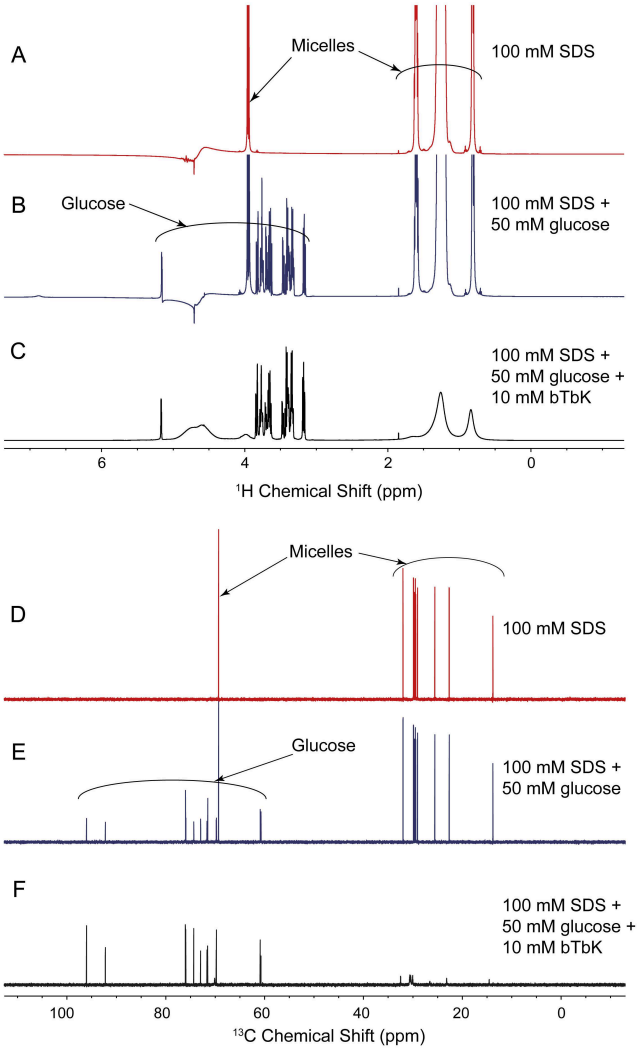
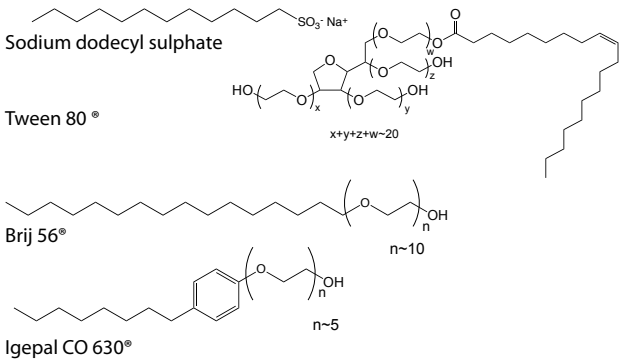


Figure 1. Incorporation of the radical bTbK into sodium dodecyl sulphate micelles (SDS) in water. ^{1}H and ^{13}C spectra of : (A, D) a solution of pure SDS micelles, (B,E) same as (A, D) with the addition of 50 mM glucose, (C,F) same as (B,E) with the addition of 10 mM of bTbK. Notably the PRE induced by the paramagnetic radical strongly affects only the micelle resonances. The resonances of the glucose, which is dissolved in the aqueous phase, are only minimally broadened by an outer sphere relaxation mechanism induced by the long-range effects of the radicals incorporated into the micelles. The spectrum in F appears more intense due to the faster ^{13}C longitudinal relaxation induced by the micelle-incorporated radical.

bTbK can also be solubilized in the glycerol/water mixture typically used for DNP experiments (d_8-Glycerol:D_2O:H_2O 60:30:10) at a concentration of around 17 mM using 540 mM SDS. L-Alanine (50 mM) was added to monitor the DNP enhancements obtained from this mixture both on ^{1}H and ^{13}C.

^{1}H DNP enhancements were monitored with direct ^{1}H and ^{13}C cross-polarization (CP) experiments performed at 400 MHz (9.4 T) and ~105 K. This bTbK/SDS system yielded DNP enhancements of ε = 17. This result indicates that the solubilisation in micelles preserves the glassy nature of the mixture and the DNP activity of the biradical. It is also worth noting that the enhancements reported here are not directly comparable with those reported on the bTbK/SOS system,[11] since those results were obtained at a lower field and lower temperature (5 T, 83 K), and using a deuterated surfactant.



Scheme 1. Molecular formulae of the amphiphiles used here.

The past work on solubilisation of radicals in micelles[12] has shown that depending on the structure of the radical, the paramagnetic center can be incorporated in the lipophilic part of the micelle or can spend some time on the micelle surface.[12c] EPR measurements showed that radicals with larger hydrophobic surfaces are generally more embedded inside the lipophilic core of the micelle, but the nitroxide moieties can still be partially exposed in the surface.[12e,13] For a given overall radical concentration, different amphiphiles may also prevent solvent-radical interactions, as well as radical aggregation inside the micelles, to different degrees, which would be reflected in the DNP enhancements. In water, SDS micelles generally have a radius on the order of 17-22 Å, with about ~65 molecules per micelle on average.[14] The micelle is large enough to incorporate bTbK (~15 Å long), even though its size might be slightly reduced in the glycerol/water environment.[15] Conversely, SDS micelles are probably not large enough to completely incorporate larger radicals such as TEKPOL, which yield much better DNP performance in conventional cases. Furthermore, SDS, SOS and other analogous anionic surfactants are not actually particularly suitable for biological applications of DNP NMR because of their strong interaction with biomolecules, especially at the high surfactant concentrations needed here.

We thus turned our attention to neutral amphiphiles that are more compatible with biological systems. Neutral amphiphiles have low cmc and can form larger micelles, and should be able to solubilize larger molecules, since the micelle dimension is not limited by the stabilization of the charged surface, as in SDS.We looked at the performance of the surfactants Tween-80®, Brij-56® and Igepal CO 630® with the radicals bTbK, bCTbK, BDPA and TEKPOL (Scheme S1, Table S1) leading to the conclusion that the surfactant Tween-80 is a good alternative to SDS. Tween-80 is the common brand name for the non-ionic amphiphile polysorbate 80 (Scheme 1). Tween-80 is a surfactant with emulsifier properties that is widely used as a food additive and a drug excipient. It stabilizes aqueous formulations of pharmaceuticals (such as eye drops, etc.) and proteins, preventing protein aggregation.[16a] It is

thus expected not to significantly perturb a biological system under investigation.[16b] Tween-80 has good solubility both in water and in lipophilic environments (methanol, toluene, ethylacetate). In water the reported cmc is only 12 μM, leading to a lower surfactant concentration than SDS to form micelles.

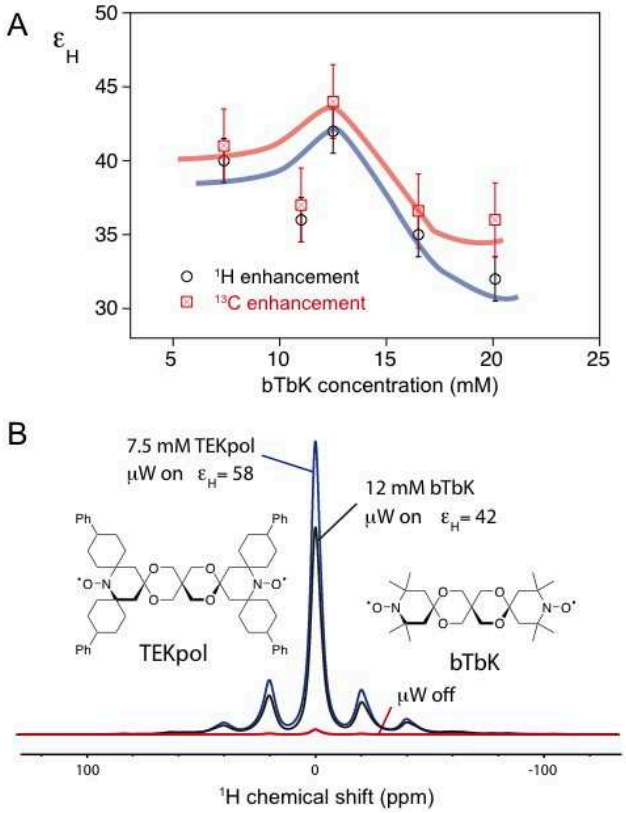


Figure 2. A) ^{1}H DNP enhancements as a function of bTbK concentration in 190 mM Tween-80 micelles in d_8-Glycerol:D_2O:H_2O 60:30:10. B) Comparison of the ^{1}H enhancement obtained for 7.5 mM TEKPOL and 12 mM bTbK. The ^{1}H spectra were obtained with direct excitation in a rotor synchronized echo experiment (400 MHz, MAS frequency = 8.0 kHz, T ~105 K), and a 2.5 μs π/2 pulse. One rotor period was used in each echo delay. The acquisition time was 5.12 ms over a spectral window of 200 kHz. The recycle delay was 3.0 s.

bTbK solutions can be easily prepared up to ~30 mM in a solution of d_8-Glycerol:D_2O:H_2O 60:30:10 with only ~190 mM of Tween-80 (see experimental section). Figure 2 shows the trend of the ^{1}H DNP enhancement observed at 400 MHz on such aqueous bTbK solutions with concentrations ranging from 7 to 20 mM. At a concentration of 12 mM a ^{1}H enhancement of ε_H = 42 is observed for the bulk solvent, and a similar value of $\varepsilon_{C\text{-}CP}$ = 44 for ^{13}C detection on L-proline dissolved in the H_2O phase. These enhancements are analogous to those observed when bTbk is dissolved in tetrachloroethane (TCE),[4d] which indicates that bTbK does not aggregate in micelle and that the glassy matrix is preserved in presence of the surfactant.

Other radicals that are insoluble in water, such as TEKPOL or BDPA, can also be solubilized in analogous conditions. In particular TEKPOL can be solubilized up to concentrations of 7-8 mM in the same Glycerol/water/Tween-80 mixture used above, yielding ^{1}H DNP enhancements of ε_H = 58 (Figure 2B). This enhancement is lower than the enhancement observed in TCE (at 16 mM), which could be due to the lower concentration of TEKPOL here, or because the enhancement is affected by the increased proton/deuterium ratio since here we used a fully protonated surfactant. We anticipate that large additional gains in enhancement could be obtained from using deuterated surfactant. Figure 3 reports the enhancements observed using several different surfactants to solubilize TEKPOL, the nature of the surfactant clearly has a large effect, and Tween-80 yields the best performance so far, probably due to better solubilisation which prevents radical aggregation inside the micelle. Further investigation of the detailed role of the amphiphile, and optimisation of these systems in terms proton/deuterium ratios, will be the subject of future work.

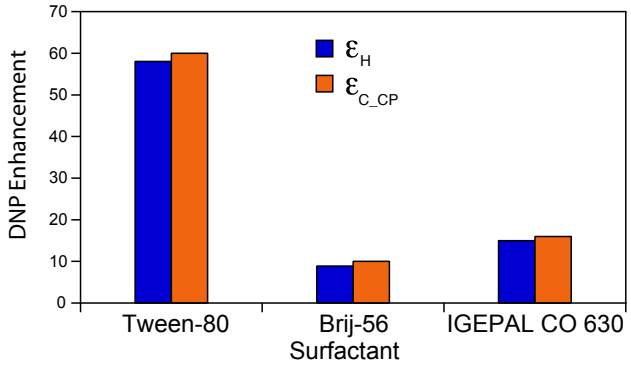


Figure 3. DNP enhancements of 7.5 mM TEKPOL with three different surfactants. ^{1}H enhancements were measured with direct excitation and a rotor synchronized echo, while the ^{13}C enhancement was measured through a ^{1}H-^{13}C CP experiment using ^{13}C labelled L-proline as an internal probe.

Finally, the carbon-centered monoradical trityl derivative BDPA also solubilizes well in Glycerol/water/Tween-80, with concentrations up to 18-20 mM possible with ~200 mM Tween-80. This radical is particularly relevant to very low temperature dissolution DNP experiments.[17] As a proof of principle, under the conditions used here (400 MHz, ~100 K, conc. 14 mM) a DNP effect of ε_H = 4.8 is obtained, which is analogous to the ε_H = 4.8 measured in TCE (radical concentration 32 mM)..

Conclusions

The solubilisation of hydrophobic polarizing agents in micelles relieves many of the constraints on the design of DNP polarizing agents for applications in aqueous environments. The approach is general, and can be adapted to different radicals without losing DNP efficiency as compared to organic solvents. Here we have shown that high DNP enhancements can be obtained for the current best hydrophobic bi-radicals (TEKPOL) in combination with the neutral surfactant Tween-80.

Notes and references

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(ESI) available: Materials and Methods, Scheme S1, Table S1. See DOI: 10.1039/c000000x/

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SUPPLEMENTARY INFORMATION

Hydrophobic Radicals Embedded in Neutral Surfactants for Dynamic Nuclear Polarization of Aqueous Environments at 9.4 Tesla.

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Materials and Methods.

General Information. The surfactants Tween-80®, Brij® C10 (Brij-56), IGEPAL® CO-630, and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich. L-Alanine was purchased from ACROS Organics, α-D-Glucose was purchased from Sigma-Aldrich, Uniformly ^{13}C-labelled L-proline was purchased from Cambridge Isotope Laboratories. The radical BDPA was purchased from Sigma-Aldrich, while bTbK,[1] bCTbK,[2] and TEKPol[3] were prepared following the synthesis already reported in the literature.

DNP-NMR Methods. All DNP-enhanced NMR experiments were performed using a solid-state 400 MHz DNP-NMR spectrometer designed by Bruker-Biospin[4]. This system consists of a wide-bore 9.4 T magnet ($\omega_H/(2\pi)$ = 400.3 MHz, $\omega_C/(2\pi)$ = 100.7 MHz) with a Bruker Avance III spectrometer console, and is equipped with a double/triple resonance 3.2 mm low-temperature CP-MAS probe. DNP is achieved by irradiating the sample with high-power microwaves at a frequency of 263 GHz that are generated by a gyrotron and are delivered to the sample by a corrugated wave-guide (~5 W of power reaching the sample). The gyrotron operates continuously during the DNP-enhanced experiments (stability of better than ±1%). Sapphire rotors (with ZrO_2 caps) were used for optimal microwave penetration. Spinning frequencies were regulated to 8.0 kHz ± 2 Hz. The sample temperatures were ≈ 105 K. The chemical shifts are referenced to TMS at 0 ppm.
^{1}H 1D direct excitation experiments were acquired with a rotor synchronized spin echo in order to suppress the background signals. The pulse sequence was: $\pi/2 - \tau - \pi - \tau -$ acquisition, $\pi/2$ and π hard pulses were calibrated at 2.5 μs and 5.0 μs (100 kHz), respectively. The τ echo-delays were set to 1 rotor period. Experiments were acquired over a spectral window of 200 kHz, with an acquisition time of 5.12 ms, and a recycle delay of 3.0 s. Standard cross-polarization (CP) was used for the acquisition of 1D ^{13}C spectra, the recycle delay between scans was 2.0 s in all experiments. The ^{1}H $\pi/2$ pulse length was 2.5 μs

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(ν_1 = 100 kHz). A linear amplitude ramp (from 100% to 50% of the nominal RF field strength) was used for the ^{1}H channel, with a 2.0 ms contact time (τ_{CP}), and a nominal RF-field amplitude (ν_1) of 88.4 kHz for ^{1}H and 58.1 kHz for ^{13}C. SPINAL-64[5] proton decoupling was applied during the acquisition of the ^{13}C signal with ν_1 = 100 kHz. The 1D fid ^{13}C acquisition time was 25.3 ms for 1024 complex points. 1D spectra were processed using exponential window functions with a linebroadening of 200 Hz for ^{13}C and 400 Hz for ^{1}H spectra.

Solution-NMR Methods. Liquid state NMR spectra were acquired on a 600 MHz Bruker instrument (14.1 T) equipped with a ^{1}H/^{13}C/^{15}N cryoprobe and an Avance III Bruker console. The samples were prepared in aqueous environment using H_2O/D_2O 90/10 as solvent. The temperature was stabilized to 298 K.

The ^{1}H 1D spectra were acquired with an excitation sculpting water suppression sequence,[6] with selective π pulses on water of 2000 μs, non-selective π pulses of 20.4 μs and a short π/2 excitation pulse of 1.0 μs. Each spectrum was recorded accumulating 128 scans with 852 ms of acquisition time and 1.15 s of recycle delay. The spectra were processed with an exponential windows function of 0.3 Hz.

The ^{13}C 1D spectra were acquired with a direct excitation double-echo experiment to suppress the probe background and to remove the baseline distortion. The sequence was π/2 — τ — π — 2τ — π — τ — acquisition: the π/2 and π hard pulses were calibrated at 12.0 μs and 24.0 μs, respectively; the τ echo-delays were optimized to 50 μs each. Waltz-16 ^{1}H-decoupling at 3.12 kHz was applied during acquisition and a weaker ^{1}H-decoupling at ~2 kHz was kept during the recycle delay. Each experiment was acquired accumulating 128 scans with 865 ms of acquisition (64 k real points) and 2.0 s of recycle delay. The spectra were processed with an exponential windows function of 0.3 Hz.

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Sample Preparation.

bTbK dissolved in sodium dodecyl sulphate aqueous solutions. A solution of 100 mM sodium dodecyl sulphate (SDS) and 50 mM α-D-Glucose solution was prepared in H_2O/D_2O 90/10. The dissolution of bTbK was performed by directly stirring a weighted amount of bTbK powder with the previously prepared 100 mM SDS solution until complete dissolution.

bTbK dissolved in glycerol/water/SDS solution. A solution of 400 mM sodium dodecyl sulphate (SDS) and 50 mM L-alanine was prepared by dissolving the weighted powder in a d_8-Glycerol:D_2O:H_2O 60:30:10 mixture. bTbK was than dissolved by directly stirring the radical powder in the above prepared glycerol/water/SDS solution by gently heating the mixture at 40-50 ˚C.

Radicals dissolved in glycerol/water/surfactant solution. The used radical (bTbK, bCTbK, TEKPol, or BDPA) was first dissolved in the pure surfactant (Tween-80®, Brij® 56, IGEPAL® CO-630) by stirring the radical powder in the melted amphiphile gently heated at around 40-50 ˚C. Radical solution with a concentration of ~50 mM can thus be obtained in such a way. Then this solution was diluted up to the desired radical concentration by addition of the d_8-Glycerol:D_2O:H_2O 60:30:10 mixture. Uniformly ^{13}C-labelled L-proline was added to the solution in order to have a final concentration of 12 mM in proline to monitor the ^{13}C enhancement in CP experiments. For the case of Tween-80 and a radical concentration of 12 mM, a final surfactant concentration of ~190 mM was used.

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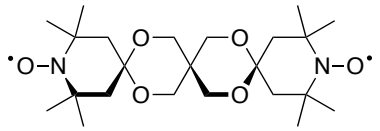
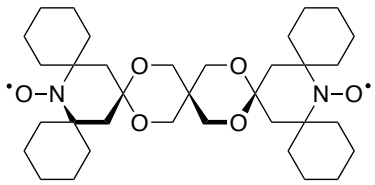


bTbK



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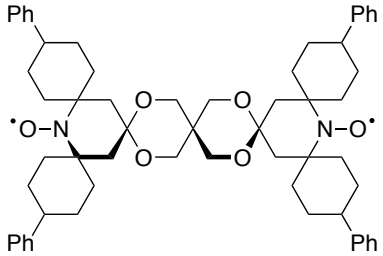
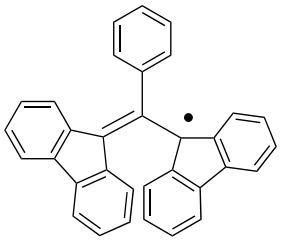


TEKPol



BDPA

Scheme S1. Molecular structures of the radicals.

SUPPLEMENTARY INFORMATION

Table S1. DNP Enhancements observed for several water-insoluble radicals solubilized in different surfactant solutions. The ^{1}H (ε_H) and the ^{13}C (ε_{C_CP}) enhancements are measured comparing experiment with and without microwave irradiation in direct excitation and ^{1}H-^{13}C CP experiments, respectively (the conditions are reported in the Materials and Methods section).

Radical	Amphiphile	Conc. (mM)	ε_H	ε_{C_CP}
bTbK	SDS	10	17	17
bTbK	Tween-80	12	42	44
bCTbK	Tween-80	12	22	24
BDPA	Tween-80	14	4.8	-
TEKPol	Tween-80	7.5	58	59
TEKPol	Brij-56	10	8.9	10
TEKPol	IGEPAL CO-630	10	15	16

SUPPLEMENTARY INFORMATION

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